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Bright field versus phase contrast microscopy in activated sludge image acquisition methodologies

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Abstract

Monitoring activated sludge processes by microscopic observations and image analysis methodologies is a well established technique with the utmost importance for microbial community characterization. Furthermore, image processing and analysis is currently considered a powerful tool to identify and quantify biomass morphological and physiological changes. In the present work, image processing and analysis methodologies were used to determine aggregates and protruding filaments contents and morphology using both bright field and phase contrast microscopy. The obtained results showed that the simpler and less expensive bright field microscopy also provided the best overall results.

1 Introduction

An activated sludge system includes a complex ecosystem composed of different types of microorganisms such as protozoa, metazoan and filamentous, zoogleal and other bacteria. A good balance between the different types of microorganisms is essential to guarantee good settling properties and a clear supernatant which can be performed by visual inspection under an optical microscope coupled to automated image analysis methodologies. As a matter of fact, in recent years, activated sludge processes have been already monitored through microscopic observations for aggregates contents and morphology and protruding filamentous bacteria contents determination (da Motta *et al*., 2001, Amaral and Ferreira, 2005, Jenné *et al*., 2007).

Automated image analysis is considered to be a feasible method to characterize quantitatively both aggregated and filamentous bacteria, with two image acquisition methodologies standing out: phase-contrast microscopy as proposed in the works of Cenens *et al*., 2002 and Jenné *et al*., 2007 among others; and bright field microscopy as in the works of da Motta et al., 2001, and Amaral and Ferreira, 2005. In comparison, bright field microscopy is the cheapest and simplest activated sludge examination methodology, whereas the phase-contrast microscopy requires more expensive equipment and a skilled operator. Furthermore, the inner nature of the phase contrast microscopy causes the aggregates borders to become ill-defined, as the objects hallo hinders the assessment of their boundaries. However, this methodology presents the advantage of a more precise determination with respect to the protruding filamentous bacteria contents. As a matter of fact, the high transparency of the filamentous bacteria poses a contrast problem in bright field microscopy acquisition, opposite to the clear filaments/background distinction in phase contrast microscopy. Therefore, it comes as no surprise that studies have already been

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performed using bright field acquisition methodologies to survey aggregated biomass and phase contrast acquisition for filamentous bacteria assessment (Amaral, 2003).

In order to determine the best image analysis acquisition methodology of activated sludge samples, the present work aims to determine both protruding filamentous bacteria and aggregated bacteria contents using, on one hand, bright field microscopy and, on the other, phase contrast microscopy.

2 Material and Methods

Experimental study

The biomass used in this study was collected from the aeration basins of seven wastewater treatment plants, treating domestic effluents, located in the North of Portugal. Samples were taken to perform microscopic observations, in order to estimate the contents of the microbial aggregates and protruding filamentous bacteria by image acquisition and analysis. For each sample, the biomass content (TSS) was determined by dry weight (APHA *et al*., 1989).

Image Acquisition

A volume of 25 µL was placed on a slide and covered with a 20x20 mm cover slip for visualization and image acquisition in bright field and phase contrast microscopy. Roughly 200 images were acquired per sample to obtain significant data for both acquisition methodologies.

Bright field microscopy: Images were acquired in a Leitz Laborlux S optic microscope (Leitz, Wetzlar), with 100x magnification, coupled to a Zeiss AxioCam (Zeiss, Oberkochen). Image acquisition was performed in 1300 x 1030 pixels and 8-bit format through the commercial software Axio Vision 3.1 (Zeiss, Oberkochen).

Phase contrast microscopy: Images were acquired in a Diaphot 300 microscope (Nikon Corporation, Tokyo) with 100x magnification, coupled to a Sony CCD AVC–D5CE (Sony, Tokyo) grey scale video camera. The images were acquired in 768×576 pixels and 8-bit format by a Data Translation DT 3155 (Data Translation, Marlboro) frame grabber using the commercial software package Image Pro Plus (Media Cybernetics, Silver Spring).

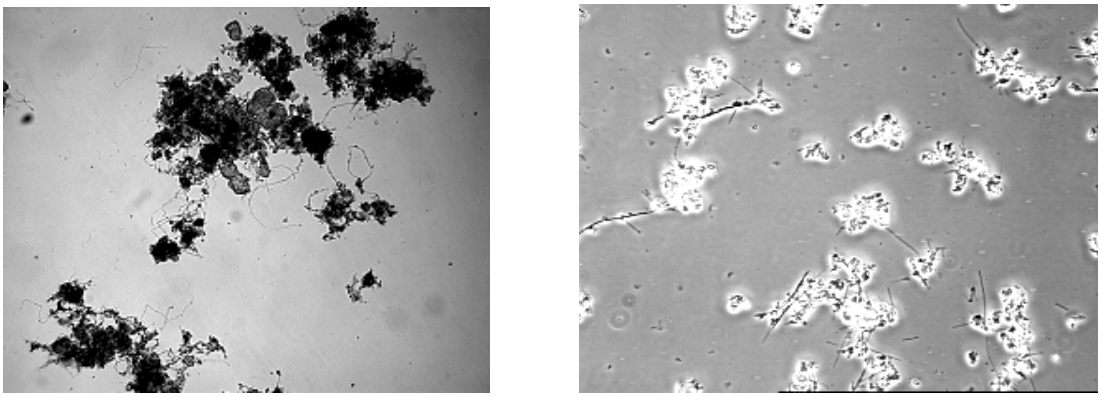

Figure 1. Bright field and phase contrast images.

Calibration from pixels to the metric unit dimensions was performed by means of a micrometer slide.

Image Processing and Analysis Methodology

The aggregates and filaments contents and morphological descriptors were determined by means of image processing and analysis methodologies developed in Matlab 7.3 (The Mathworks, Inc., Natick) language adapted from a previous program developed by Amaral and Ferreira (2005).

The image processing and analysis methodology is divided into two stages. Primarily, the image processing program determines and saves both the aggregated and protruding filamentous biomass binary images. Secondly the image analysis program proceeds to the aggregates and filaments contents and morphological parameters determination.

Image Analysis Parameters

Supported on the previous study of Amaral and Ferreira (2005) 4 parameters were determined, either directly from the image analysis program, either in association with the sludge physical properties, for a total of 131 different samples. Total aggregates area (TA), total filaments length (TL), total filaments length per volume (TL/Vol), total aggregated area per volume (TA/Vol), and total filaments length per total aggregates area (TL/TA) were determined alongside the total filaments length per total suspended solids (TL/TSS) characterizing the aggregates and filaments dynamics. Those parameters were used to establish the best acquisition methodology for both aggregated and protruding filamentous bacteria. A more detailed description of each parameter can be found in Amaral and Ferreira (2005).

3 Results and Discussion

The results herein depicted in Figure 2 revealed a similar trend for both acquisition methodologies with respect to the four studied parameters. However, it is also notorious some differences regarding the absolute value of the TA/Vol (Figure 2a) and TL/TA (Figure 2c) throughout the whole of the data points and in TL/Vol (Figure 2b) and TL/TSS (Figure 2d) in some samples.

Analyzing the aggregated biomass contents, in terms of TA/Vol (Figure 2a), throughout the data points the values obtained for the phase contrast methodology were higher than for the bright field methodology. As a matter of fact, the full data set revealed a 42% increase on the TA/Vol values for the phase contrast acquisition. Given the poorer representation of the objects boundaries in phase contrast microscopy, it seems safe to conclude that this methodology overestimated the aggregated biomass contents considerably.

With respect to the protruding filamentous bacteria contents, in terms of TL/Vol (Figure 2b), for a significant part of the analyzed data, phase contrast and bright field methodology provided similar results. However, for most of the data points there seems to take place a slight overestimation for the bright field microscopy. This fact resulted in an 18% increase on the TA/Vol values for the bright field acquisition. Given the better filaments/background contrast obtained by the phase contrast microscopy, these results were not expected. However, these results may be explained by the fact that the activated sludge samples analyzed in this work presented low protruding filamentous bacteria contents, and that the aggregates area is overestimated in phase contrast. That being the case, the filaments true length would be shortened by the halo of the aggregates in phase contrast, which is a pressing matter in the studied activated sludge, which presented low (and short) protruding filamentous bacteria.

The difference between bright field and phase contrast acquisition is also quite clear when observing the TL/TA (Figure 2c) which comprises only image analysis information (expressing filaments per aggregates area). Bright field microscopy seems to provide higher results than phase contrast which is due to the highest recognition of filaments and lowest aggregates detection. Therefore, it comes as no surprise that, the analysis of the full data set revealed TL/TA values for the phase contrast acquisition that were half of the bright field values.

According to Schuler and Jassby (2007), expressing filaments contents per mass (TL/TSS) is probably the most useful way for comparing filaments contents data from different studies and/or from samples with different biomass concentrations. Given the fact that such concentrations can vary greatly from one system to the other this approach normalizes filaments contents to biomass. Regarding this parameter behavior (Figure 2d) with biomass

normalization, a similar trend between the bright field and phase contrast methodologies arises when compared to the TL/Vol analysis. The main difference relies on the fact that the phase contrast TL/TSS ratio underestimation slightly increased to 22% instead of 18%.

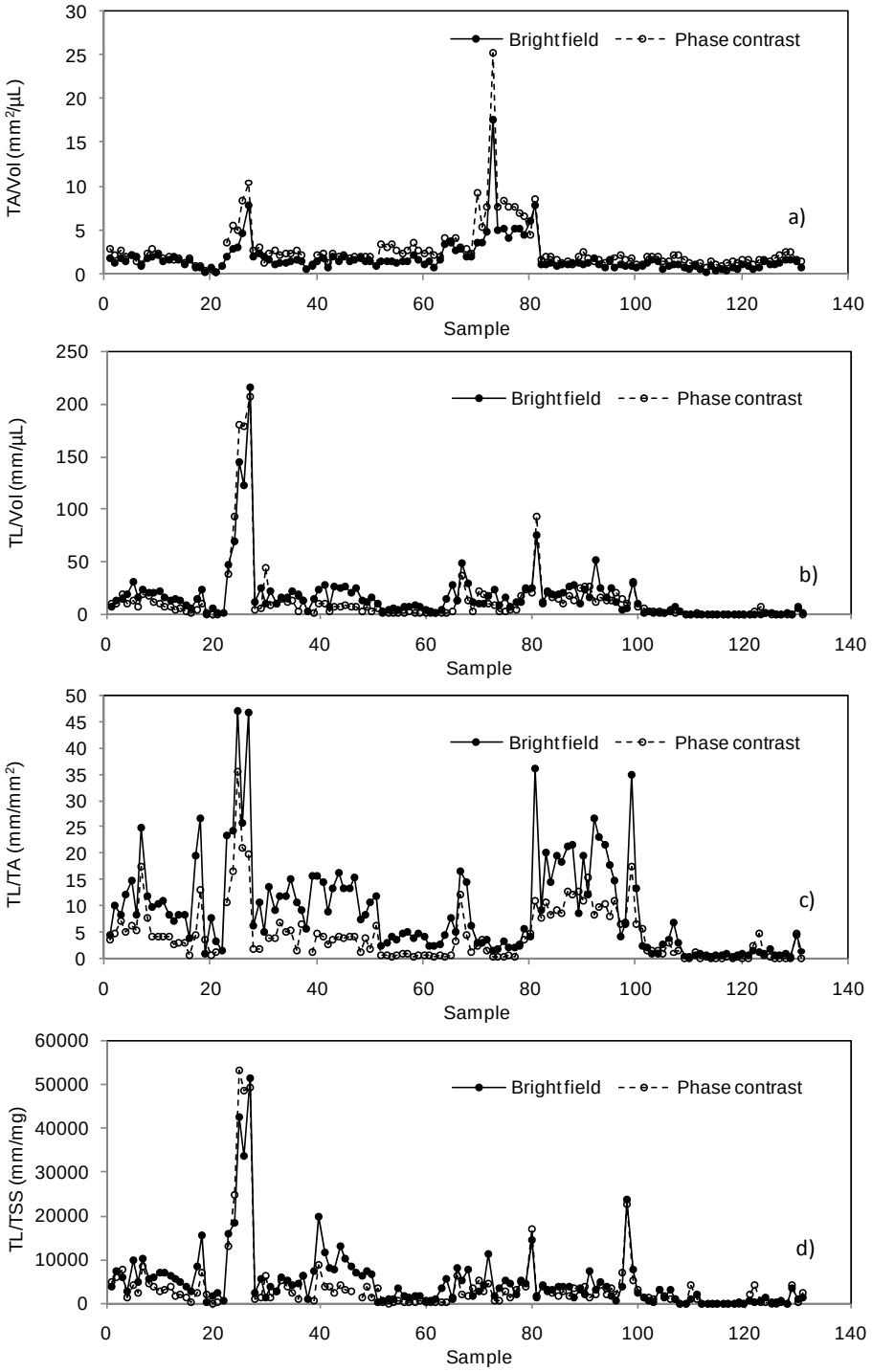

Figure 2. Bright field and phase contrast results for TL/Vol (a), TA/Vol (b), TL/TA (c) and TL/TSS (d) considering 131 samples.

Regarding the behaviors presented in Figure 2, it seems clear the existence of a correlation between the results for bright field and phase contrast microscopic acquisition. Bearing this in mind, were seek the relationships between the bright field and phase contrast results, in order to establish the best acquisition methodology for both aggregated and protruding

filamentous bacteria. Figure 3 represents the obtained relationships between bright field and phase contrast methodologies.

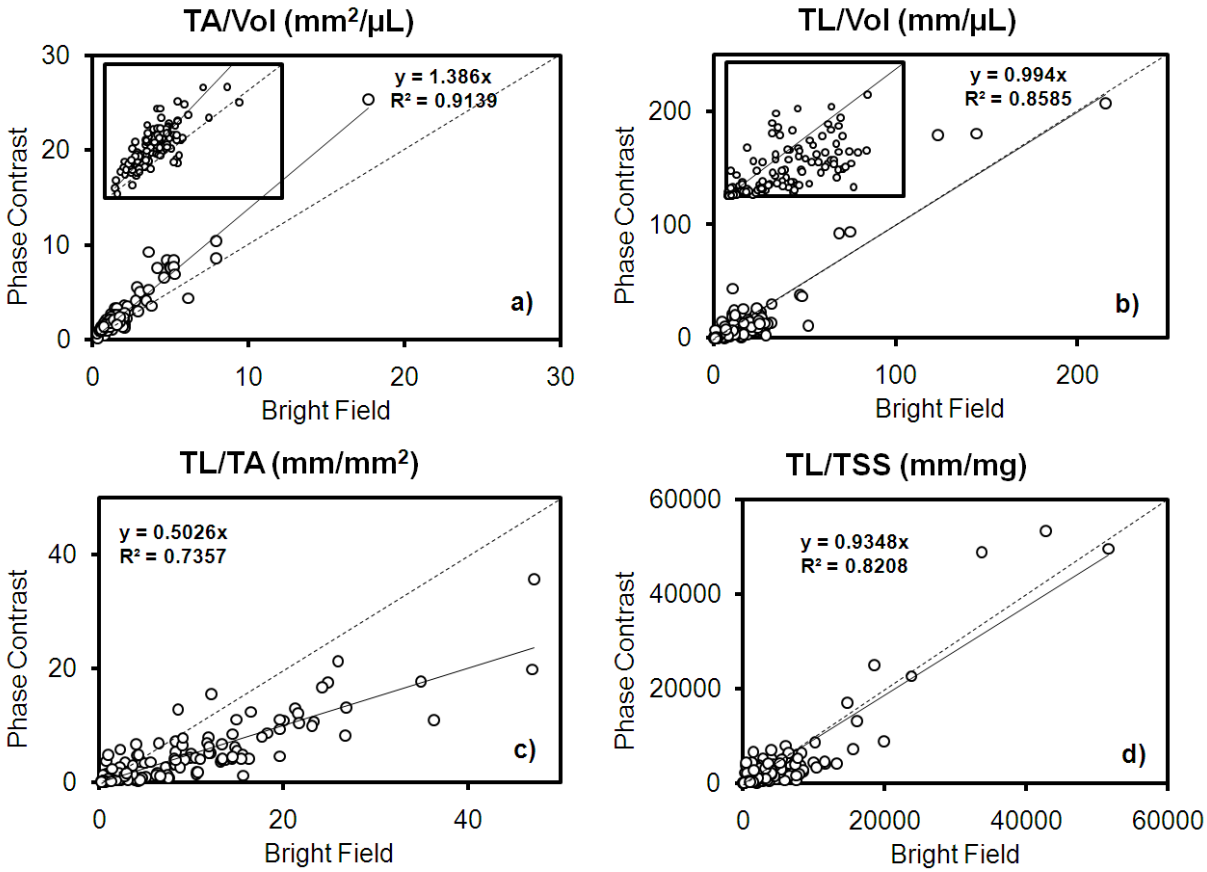

Figure 3. Bright field and phase contrast regressions for TL/Vol (a), TA/Vol (b), TL/TA (c) and TL/TSS (d).

The results herein reported in Figure 3a, revealed a satisfactory correlation coefficient (0.956) for the TA/Vol between bright field and phase contrast assessment. Again, it was possible to infer that the phase contrast methodology overestimated the aggregates detection with respect to the bright field acquisition method. Furthermore, the obtained trend line for the TA/Vol estimation points towards a global overestimation around 39% which is not far apart from the 42% obtained in the previous point to point analysis.

With respect to the protruding filamentous bacteria recognition (Figure 3b), the obtained correlation (0.927) was not as satisfactory as for the TA/Vol and a higher dispersion between bright field and phase contrast assessment was noticed. Furthermore, the trend line pointed to a global correspondence between the two methodologies against the phase contrast underestimation of 18% in the point to point analysis. The reason between such discrepancies may be attributed to the importance of the larger TL/Vol points with respect to the correlation determination. As a matter of fact, the lower values on larger filaments contents of the bright field methodologies, compensates, at some extent, the larger values obtained for smaller contents. It should be noted that, in larger filaments contents, the advantage of the phase contrast methodology on filamentous bacteria recognition is less hindered by the ill defined aggregates borders, leading to a more precise (and higher) filaments determination. All things considered, the correspondence found between the two methodologies allows inferring that the bright field acquisition does not lead to overall significant errors with respect to the protruding filamentous bacteria determination. However care should be taken in analyzing the results of bright field acquisition from activated sludge with high filamentous contents.

Observing Figure 3c, a considerable dispersion between bright field and phase contrast assessments was detected and a poor 0.858 correlation coefficient for the TL/TA ratio was achieved. This result might be due to the cumulative sum of impreciseness both in terms of TL and TA, resulting also in a strong underestimation of this parameter in phase contrast acquisition. This conclusion is further emphasized by the 0.50 slope obtained. This results in a global 50% underestimation of the TL/TA ratio by the phase contrast methodology which is in accordance with the 50% reduction in the previous point to point analysis.

Regarding the TL/TSS ratio results (Figure 3d), a non completely satisfactory correlation coefficient of 0.906 was obtained meaning a global underestimation for the phase contrast of 7% (0.93 slope). Again, a discrepancy was found between the 7% underestimation pointed by the trend between the two methodologies against the phase contrast underestimation of 22% in the point to point analysis. The reason for this behavior is directly related to the protruding filamentous bacteria estimation differences found in the two methodologies, and the same caution must be applied in the TL/TSS ratio as in the TL/Vol assessments.

4 Conclusions

This study clearly demonstrated that, with respect to the protruding filamentous bacteria, the bright field acquisition results mimic, at a certain extent, the phase contrast results. However, the inverse relationship does not hold true for the aggregated biomass contents assessment for the phase contrast acquisition. In fact, it was found that this methodology overestimates by approximately 40% the results obtained by the bright field approach. Thus, considering the advantages and disadvantages of each acquisition methodology and the obtained results, the bright field microscopy proved to be not only more simple and inexpensive but also provided the best overall results.

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